Physiological Characterization of Drought Stress Response and Expression of Two Transcription Factors and Two LEA Genes in Three *Prunus* Genotypes

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ABSTRACT

Global warming has led to a progressive decrease in rainfall, which is reflected by a reduction of water resources in the soil and a negative effect on crop production in Mediterranean areas. Under drought stress, many plants react by inducing a different series of responses at both physiological and molecular levels, allowing them to survive for a variable period of time. Therefore, in order to understand the response of roots to drought conditions, the genotypes peach × almond 'Garnem' [*P. amygdalus* Batsch × *P. persica* (L.) Batsch] and their progeny, the hybrid 'P.2175' × 'Garnem'-3 and OP-'P.2175' (*P. cerasifera* Ehrh.) were subjected to a period of water deficit. Drought conditions with a subsequent re-watering period were tested for potted plants for one month. Stomatal conductance and leaf water potential were measured to monitor the plant physiological responses. Significant differences among the drought stress and drought stress recovery treatments and among the genotypes were observed. In addition, four genes related to the ABA biosynthesis pathway were studied for their expression by RT-qPCR: an AN20/AN1 zinc finger protein (*ppa012373m*); a bZIP transcription factor (*ppa013046m*); a dehydrin (*ppa005514m*) and a LEA protein (*ppa008651m*). Their expression profiles correlated with our physiological results of drought response, being higher in roots than in phloem tissue. In general, the expression of the four studied genes was higher after 15 days under drought

conditions. Under drought and recovery conditions, the zinc finger and bZIP transcription factors showed significant differences in their relative expression levels from LEA and dehydrin. These results suggest the role of LEA and dehydrin in the regulatory response to drought stress in *Prunus* genotypes. Therefore, the dehydrin and the protein LEA might be potential biomarkers to select rootstocks for tolerance to drought conditions.

Keywords ABA, LEA protein, qPCR, Transcription Factor, Water deficit.

1. INTRODUCTION

Stress can be defined as a physiological deviation from normal plant functions that can damage or cause irreversible damage to the plant (Nagarajan, 2010), negatively affecting crop growth and yield. Drought stress is one of the biggest problems in agriculture, especially in arid and semi-arid climates (Bartels and Sunkar, 2005) in the Mediterranean region where water availability is the most important factor for plant survival. Since Mediterranean countries are the main stone fruit producers (FAO, 2014), the use of adapted rootstocks is necessary for such limited edaphoclimatic conditions. Currently, the challenge in rootstock breeding programs is the combination of abiotic tolerances in a new generation of interspecific hybrids resulting from the cross of almond × peach hybrids by plum genotypes. Peach × almond hybrids such as 'Garnem', 'Felinem' and 'Monegro' (which come from the cross 'Garfi' almond × 'Nemared' peach) show good vigour, nematode resistance, and adaptation to calcareus soils (Felipe, 2009). Myrobalan plums such as 'P.2175' provide a wide spectrum of root-knot nematode resistance (Rubio-Cabetas et al., 2000) and tolerance to waterlogging (Amador et al., 2012).

During the stress period, plants undergo some morphological and physiological changes due to hormones such as abscisic acid (ABA) and ethylene (Bruce et al., 2002; Munns, 2002). ABA accumulation under water deficit conditions activates different genes linked to stress (Narusaka et al., 2003). The ABA-inducible genes have *cis*-elements in their promoter regions including *ABA-responsive elements* (ABRE) (Yamaguchi-Shinozaki and Shinozaki, 2005). The activation of these elements through different transcription factors (TFs) ABA-responsive element binding proteins, such as ABI/ABF/AREB/bZIP families (Hossain et al., 2010; Qin et al., 2014; Uno et al., 2000), induces the expression of many downstream genes involved in drought tolerance or enzymes involved in the catalysis of low molecular weight osmolytes (Beck et al., 2007). Jakoby et al. (2002) identified 75 different bZIP TFs divided in ten groups. One of them is the Group S, whose TFs are transcriptionally activated after stress treatment, such as drought (Jakoby et al., 2002). *AtbZIP53* TF, found inside this group S, functions as transcriptional

activator of the *ProDH* gene in *Arabidopsis* (Satoh et al., 2004) with leads to the decomposition of proline accumulated during dehydration period (Satoh et al., 2004; Yoshiba et al., 1997). In addition to these TFs, among others, there are genes belonging to the *Stress Associated Protein* (SAP) genes family which encodes proteins containing A20/AN1 zinc-finger domains (Ben Saad et al., 2010). Proteins with zinc-fingers A20/AN1 type are described in numerous species such as *Oryza sativa* (Vij and Tyagi, 2006), *Populus trichocarpa* (Jin et al., 2007), and *Aeluropus littoralis* (Ben Saad et al., 2010) among others, suggesting an important role in abiotic stress responses in plants, such as cold, salt, dehydration, heavy metals, submergence, wounding as well as stress hormone abscisic acid (Vij and Tyagi, 2006).

After the early response to stress of TFs, the expression of different target genes coding proteins, such us chaperones, late embryogenesis abundant (LEA) proteins, osmotin, mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes, and various proteases take place (Shinozaki and Yamaguchi-Shinozaki, 2007). In particular, protecting function of LEA proteins has been widely demonstrated in literature. For example, overexpression of *HVA1* confers drought tolerance in transgenic rice (Babu et al., 2004; Chen et al., 2015). LEA-type proteins play a main role in storage of seeds as well as acclimation and adaptive response to stress processes conferring molecular protection of cellular components during abiotic stress (Battaglia et al., 2008; Xiao et al., 2007) by the influence of ABA concentration changes (Hong-Bo et al., 2005). ABA accumulation produced by drought stress induces the activation of *ABA responsive elements* (ABRE) *cis*-elements regulating the transcription of most *LEA* genes (Hundertmark and Hincha, 2008), which are organized in several groups depending on sequence similarity, and therefore, on functionality (Battaglia et al., 2008). One of them is group II, known as D-11 family whose proteins are called dehydrins (Allagulova et al., 2003). Dehydrins have been studied in several species (Lopez et al., 2001, 2003; Yamasaki et al., 2013), and more particularly in woody plants (Artlip and Wisniewski, 1997; Bassett et al., 2009; Velasco-Conde et al., 2012; Vornam et al., 2011; Wisniewski et al., 2009, 2006). Up to date, three dehydrin genes (*Ppdhn1*, *Ppdhn2* and *Ppdhn3*) have been described in peach confirming its induction by

drought and its implication in cold acclimation (Artlip and Wisniewski, 1997; Bassett et al., 2009; Wisniewski et al., 2006).

Due to the complexity of drought tolerance mechanisms, improvements in the breeding of this trait have been slow (Tuberosa and Salvi, 2006). New cultivars obtained, showing drought tolerance, have been mostly released in classical breeding programs. Gene introgression from other species through interspecific hybridization has been used in many breeding programs: crossing almond × apricot, but also peach with wild species such as *P. webbii*. This gene introgression led to the production of drought-tolerant rootstocks (Felipe, 2009; Martínez-Gómez et al., 2003). A variety of studies have been undertaken in order to understand the physiological and genetic basis of the hydric stress response on fruit trees (Basile et al., 2003; Karimi and Yadollahi, 2012; Liu et al., 2012), and also, on interspecific hybrids from *Prunus* genus (Jiménez et al., 2013; Sofo et al., 2005; Xiloyannis et al., 2007). Furthermore, molecular biology as well as genomics led to the identification of candidate genes. In peach, different genes that encode for dehydrins have been identified (Artlip et al., 1997; Bassett et al., 2009; Wisniewski et al., 2006). Alimohammadi et al. (2013) categorized five candidate genes responsive to water-deficit stress and emphasized the importance of starch synthesis, sugar and ABA in *P. scoparia*. More recently, improvements in sequencing and genotyping techniques provide reference genomes in *Prunus* genus, such as peach (Verde et al., 2013) and Japanese apricot (Zhang et al., 2012), representing a new tool for breeding. Molecular studies mainly focused on transcriptomics, have led to rapid generation of information about all the genes expressed under drought conditions in a particular genotype. RNA-seq analysis studies in Mongolian almond identified genes involved in drought response (Wang et al., 2015). In the same way, Eldem et al. (2012) identified miRNAs responsive to drought in peach by Illumina deep sequencing technology.

The objective of this study was the evaluation of the response to drought stress of three *Prunus* rootstocks by measuring genotype differences in different physiological parameters and studying the expression profiles of two TFs as well as two key genes involved in drought

tolerance. The development of drought-tolerant biological markers involved in drought stress is useful in breeding programs for the selection of more drought tolerant rootstocks.

2. MATERIALS AND METHODS

2.1. Plant material and experimental conditions

The material presenting different levels of resistance against nematodes of *Meloi~~y~~dogyne* spp included two hybrid genotypes from a breeding program (EU funded project FAIR-6-CT-98-4139) and the commercial rootstock 'Garnem'. A total of 30 two-year-old plants were considered for the experiment: six plants from the almond × peach hybrid 'Garnem'; 12 plants from the 'P.2175' x 'Garnem'-3 hybrid, formerly named 'Tri-hybrid-3'; and 12 plants from the OP-'P.2175' (*P. cerasifera*). This plant material was propagated by hardwood cuttings at the CITA (Agrifood Research Centre of Aragon) facilities in Zaragoza, Spain.

These plants were placed in 20 cm diameter pots with a mix of turf, 30% coconut fibre and 20% sand. The experimental design was a two randomized block: Control and Treatment (3 plants from 'Garnem', 6 plants from 'Tri-hybrid-3' and 6 plants from OP-'P.2175' for each group). The pots were covered with black plastic in order to minimize evapotranspiration from the soil surface and to avoid the entrance of precipitation into the soil. The experiment was carried out in a shaded greenhouse located in the CITA facilities in Zaragoza (41º43'N, 0º48'W). Plants underwent a drought period beginning from July 5 to 19, 2011, followed by a re-watering period of 15 days. Before beginning the water-stress period, the water content was maintained in optimal conditions for all plants. During the treatment period, stressed plants had no water supply, whereas control plants were watered three times weekly until field capacity to maintain optimal soil water content by drip irrigation (flow dripper of 2 l/h – 15 min). After 15 days of water stress, treatment plants were re-watered supplying the same irrigation level and frequency as the control plants during 15 days more to restore the water soil conditions. The average climatic conditions during the experimental period were the following: temperature of 22.3 ºC; relative humidity of 54.8%; solar radiation of 26.9 MJ m^{-2} day^{-1}; rainfall of 0.14 mm day^{-1}; and ETo of 6.5 mm day^{-1}. (Extended environmental data are shown in Supplementary Table S1).

Samples of root and phloem tissues from each plant were collected, considering two biological replicates, from the control and treated plants on days 0, 10 and 15 during the drought stress period and on days 10 and 15 during the re-watering period. For root sampling, each plant was de-potted, sampled, and re-potted again until next sampling. Phloem sampling was done in each plant. Stems were cut, the bark removed and the phloem tissue isolated using a scalpel. These samples were immediately frozen at -80 ºC for subsequent RNA extraction and gene expression analysis.

2.2. Physiological characterization

2.2.1. Physiological measurements

Plant water status was determined by measuring the Leaf Water Potential (LWP) twice a week at 11 am, using a Scholander-type pressure chamber (Soil Moisture Equipment Corp. Santa Barbara, CA, USA) (Scholander et al., 1964). The values of LWP were obtained from healthy old leaves from each plant of the median segment of the shoot. The selected leaves were covered with aluminium foil in order to stop transpiration before picking up them for measuring LWP. The resultant LWP data was the average of three measurements as technical replicates. Stomatal conductance (gs) was also measured twice a week at 11 am from a leaf of each plant of the median segment of the shoot with a Leaf Porometer (Decagon Devices Inc., Pullman, WA, USA). Finally, the percentage of leaf epinasty was determined in stressed plants by counting leaves without visible drought stress symptoms like leaf curling, yellowing, loss of turgidity and leaf falling, twice a week before sampling for LWP and gs according to the following equation:

$$\% \text{ Epinasty} = \frac{\text{total leaves - leaves without stress symptoms}}{\text{total leaves}} \times 100$$

2.2.2. Ash content

Three shoots with a length of approximately 35 cm were picked up, as technical replicates, from each plant during the experiment, cut into small pieces and dried at 60 ºC for 48 h in an oven. Once the wood was dried, it was ground up. Approximately 0.5 g of powder from each sample

was placed in a preheated ceramic vessel and incubated at 70 ºC overnight. Finally, samples were burnt in a muffle at 550 ºC for 24 hours. The results of the ash content were expressed as a percentage of dry mass (Glenn and Bassett, 2011).

2.3. Molecular analysis

2.3.1. RNA isolation and cDNA synthesis

Total RNA was extracted from 0.5 g of root and phloem samples as described by Meisel et al. (2005) with some modifications (Chang et al., 1993; Salzman et al., 1999; Zeng and Yang, 2002) (Supplementary Data Sheet S1). RNA integrity was verified by 1% agarose gel electrophoresis and ethidium bromide staining. Genomic DNA from RNA samples was removed by DNase I (TURBO DNA-free™, Ambion, Life Technologies, Austin, TX, USA) according to manufacturer's instructions. RNA (2500 ng) was reverse transcribed with the SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies, Carlsbad, CA, USA) in a total volume of 21 µl according manufacturer's instructions.

2.3.2. Gene expression analysis

Two microliters of a 40X diluted synthesized cDNA was used for each amplification reaction in a final volume of 20 µl. For each of two biological replicates, quantitative real-time PCR (RT-qPCR) reactions were triplicated. RT-qPCR was performed on an Applied Biosystems 7900HT Fast PCR System using PerfeCTa SYBR Green SuperMix, ROX Master Mix (Quanta Biosciences Gaithersburg, MD, USA). Specific primers corresponding to dehydrin (*ppa005514m*), the LEA protein (*ppa008651m*), the A20/AN1 zinc finger TF (*ppa012373m*) (Leida et al., 2012) and the bZIP TF were designed based on the nucleotide sequence of the *ppa013046m* gene present in the assembled and annotated peach genome (*Prunus persica* genome v1.0; http://www.rosaceae.org/) (Table 1). The amplification conditions consisted of an initial denaturation at 95 ºC for 10 min, followed by 40 cycles of 15 s at 95 ºC for denaturation, and 1 min at 60 ºC for annealing and extension. Amplification was followed by a melting curve analysis. The control reaction for RT-qPCR was performed using actin primers designed from

the available *P. persica* actin DNA sequence (Gene Bank accession number AB046952). Relative expression was measured by the standard curve procedure.

2.4. Statistical analysis

2.4.1. Physiological parameters.

For each genotype, the differences among days and within each treatment were determined using analysis of one-way variance (ANOVA) for gs, LWP, epinasty and ash content. The significant difference was assessed with Tukey's test ($p \leq 0.05$).

2.4.2. Gene expression profiles.

The statistical differences in the relative gene expression values were determined by the Student's t-test ($p \leq 0.05$) between the control (day 0) and treatment values for each gene. Furthermore, statistical differences among genotypes for each day of treatment in both phloem and root tissue were evaluated by ANOVA. The significant difference was assessed with Tuckey's test ($p \leq 0.05$).

All the statistical analyses were performed with GenStat Discovery Version 4 (VSN International, 2013)

3. RESULTS AND DISCUSSION

3.1. Physiological characterization of the drought stress response

3.1.1. Effects of drought stress on water status, stomatal conductance and leaf epinasty

During the experiment, the control plants presented constant LWP values, most of them higher than -1MPa, indicating an optimal and stable water status (Fig. 1A). These values were similar to found by Jiménez et al., (2013) in control plants of a drought experiment with four *Prunus* rootstocks. In contrast, the LWP progressively decreased in the stressed plants, confirming that this parameter depends on the soil water conditions (Davies et al., 1994; Gollan et al., 1992). Therefore, the water absorption by the roots and its movement along the plant is reduced when the water content falls (Nagarajan, 2010). In our work, this reduction was different in 'Garnem'

218 with respect to the ‘Tri-hybrid-3’ and OP-‘P.2175’ (Fig. 1A). ‘Garnem’ dramatically reduced its
219 LWP at 10 days of treatment, reaching -3.80 MPa, whereas in ‘Tri-hybrid-3’ and OP-‘P.2175’
220 this reduction was slower, showing less reduced ~~LPW~~ LWP values (-1.65 MPa and -2.57 MPa,
221 respectively). The lowest values were obtained in all genotypes after two weeks of drought,
222 which represented the period of maximum stress (Fig. 1A), when the LWP value in OP-
223 ‘P.2175’ was significantly higher than the values in ‘Tri-hybrid-3’ and ‘Garnem’
224 (Supplementary Table S2). After 10 days of re-watering, the LWP values recovered their
225 original status, reaching a water potential similar to those of the control plants (Fig. 1A) and
226 revealing a rapid recovery, as it is reflected in their leaf water potential. Similar results were
227 obtained for *Prunus* interspecific hybrids, which also reached comparable LWP values to those
228 of the control plants after 15 days of water status recovery (Sofo et al., 2005).
229 Furthermore, other significant differences between the two experimental hybrids and ‘Garnem’
230 were observed. In adequate water conditions as in day 0 and the recovery period, the LWP in
231 the two hybrids was lower than in ‘Garnem’, while the LWP was lower for the latter with
232 respect to the hybrids in drought stress conditions (Fig. 1A). Similar results were documented
233 by characterization of the drought and chlorosis tolerances in several *Prunus* tri-hybrids
234 (Xiloyannis et al., 2007). The performance of these rootstocks could be explained by the vigour
235 influence in the plant water balance (Basile et al., 2003; Hajagos and Végvári, 2013; Weibel,
236 1999). ‘Garnem’ is a vigorous rootstock (Felipe, 2009; Bielsa et al., 2015), although its vigour
237 was not reflected in the cuttings studied. Therefore, this genotype could have a greater transport
238 and water consumption under good water conditions. This corresponds to a higher LWP value
239 due to the amount of water present in the plant. In contrast, the stored water in ‘Tri-hybrid-3’
240 and OP-‘P.2175’ plants was lower, probably due to their less vigour, and hence their LWP
241 values were correspondingly low.
242 Although stomatal closure is not yet a fully understood phenomenon, LWP is one of the major
243 factors in its regulation because the stomatal aperture responds directly to maintain cellular
244 turgor (Franks et al., 1995). Rahmati et al. (2015) also observed this response. They confirmed
245 in peach that a low stomatal conductance was because of the low LWP for the three water

deficit levels studied in their work. The stomatal conductance showed a similar tendency to LWP (Figs. 1A and B). The control plants presented high gs values, although there were no significant differences among the genotypes for each day. In contrast, gs average levels decreased from 147.68 mmol m^{-2} s^{-1} on day 0 to 5.39 mmol m^{-2} s^{-1} on day 15 of treatment in the stressed plants (Fig. 1B). By 10 days of recovery, gs levels in stressed plants reached similar values as in the control plants, the hybrid genotypes showing even higher values (Fig. 1B). However, the gs value was significantly lower in 'Garnem' than in the two hybrids (Supplementary Table S2). After two weeks of recovery, 'Garnem' showed a lower gs value than the two hybrids again, but the differences in this case were not significant (Fig. 1B, Supplementary Table S2).

One possible reason can explain these observations during the drought stress period; 'Garnem' quickly consumed its water reserves, which led to a fast drop of LWP, behaving like a water spender plant (Jones and Sutherland, 1991) that absorbs all the available water in order to maintain its growth rate. In contrast, 'Tri-hybrid-3' and OP-'P.2175' would use a water saver plant strategy (Jones and Sutherland, 1991). These plants would carry on a strict stomatal control of the LWP in order to avoid the hydraulic conductivity loss. They can avoid high water deficits in the stem and maintain a minimum water level, but as a counterpart they employ a relatively risky strategy to maintain a high gs value (Vilagrosa et al., 2003; Zhang et al., 2013). This hypothesis would explain why 'Tri-hybrid-3' and OP-'P.2175' maintained a higher water level than 'Garnem' by 10 days of treatment, also showing a slightly higher gs levels, although without significant differences among them (Fig. 1A). By day 15 of treatment, the performance of 'Garnem' was similar to that of the 'Tri-hybrid-3' and OP-'P.2175'. This suggests that 'Garnem' may transform its water spender strategy into a water saver strategy once its water reserve was depleted (Jones and Sutherland, 1991; Varela, 2010). During the recovery period, 'Garnem' reached less negative LWP values than the 'Tri-hybrid-3' and OP-'P.2175' (Fig. 1A). 'Garnem' being a vigorous rootstock (Bielsa et al., 2015; Xiloyannis et al., 2007) could have a greater water transport capacity, thus this genotype would be faster in restoring the water loss in order to hold a high LWP (Zhang and Cao, 2009; Zhang et al., 2013). However, their lower

gs values indicated that the gas exchange was lower, and therefore their stomata were more sealed than the stomata of their progeny. This contradiction could be due to other factors involved in the regulation of the stomatal mechanisms in the plants (Basile et al., 2003).

In addition to the decrease of LWP and gs levels as avoidance mechanisms against drought stress, a reduction in exposed leaf area was shown by leaf curling (epinasty) until reaching loss of foliar biomass during the most severe stress time. This reduction of leaf area by epinasty and loss of biomass by leaf shedding is a typical avoidance mechanism that lowers water demand and helps to maintain the water potential in the meristems and the roots (Engelbrecht and Kursar, 2003; Kozlowski and Pallardy, 2002). A rate of 100% of epinastic leaves was reached on day 15 of treatment for all genotypes (Fig. 2). The leaf area reduction process was slower in 'Garnem' (66.7% of leaf epinasty) than in 'Tri-hybrid-3' (92.2% of leaf epinasty) and OP-'P.2175' (80.9% of leaf epinasty) on day 10 of treatment (Fig. 2). After 10 days of the recovery period, the percentage of leaf epinasty in 'Garnem' was 18.52% compared to 83.01% in OP-'P.2175' and 67.02% in 'Tri-hybrid-3', indicating a faster recovery in this genotype than in the two hybrids. In contrast, after 15 days of recovery period, the 'Tri-hybrid-3' and OP-'P.2175' showed slightly lower leaf epinasty values than those of 'Garnem' (Fig. 2), which could be related to lower gs levels presented by this rootstock (Fig. 1B). A possible explanation is that a higher new healthy leaves in 'Tri-hybrid-3' and OP-'P.2175', a higher gas exchanging capacity in these genotypes in comparison to 'Garnem'.

3.1.2. Ash content

Ash content increased with the stress level until 10 days of drought ,with 'Garnem' showing 3.8%, significantly higher than the percentage obtained by OP-'P.2175' and higher (but not significantly) than by the 'Tri-hybrid-3' (Fig. 3). Mineral accumulation in growing and transpiring tissues occurs by passive transport in the xylem (Masle et al., 1992). Thus, a higher transpiration rate correlates with a higher mineral transport to the transpiring tissues where transpiration occurs, leading to an increased ash content (Araus et al., 1998; Glenn and Bassett, 2011; Zhu et al., 2008).

The higher mineral content by 10 days of treatment in 'Garnem' could be explained by the water spender hypothesis. As a water spender plant, 'Garnem' consumes its water reserves quickly requiring a high transpiration flow along the xylem and causing a drop in the LWP (Fig. 1A). The amount of stored water would be greater in 'Garnem' than in the 'Tri-hybrid-3' and OP-'P.2175', so when the water was consumed, the mineral concentration in the tissues would also be higher. It is also true that the gs value in 'Garnem' was the lowest (Fig. 1B), which suggests a lower transpiration in this genotype. However as previously mentioned, the lack of correlation between both LWP and mineral content values in relation to the stomatal conductance could be due to other factors implicated in the stomatal closure mechanisms (Basile et al., 2003). From day 15 of treatment, the ash content significantly decreased in all genotypes, remaining stable throughout the recovery period with values that did not exceed 2.4% (Fig. 3), below the values obtained by the control plants (Fig. 1). Although 'Tri-hybrid-3' had a higher ash percentage after two weeks with an optimum water supply, this value did not differ significantly from those in the other genotypes (Fig. 3). Several previous studies have been conducted on the ash content by different authors, considering its relationship to the rate of transpiration (Masle et al., 1992), the carbon isotope discrimination ($\Delta^{13}C$) and the water use efficiency (WUE) in cereals (Araus et al., 2002, 1998; Blum, 2005; Cabrera-Bosquet et al., 2009; Merah et al., 2001), and in fruit trees (Glenn and Bassett, 2011; Glenn, 2014). In these studies, the plant material showed seasonal or annual differences with a clear response in the mineral content from the plants under drought conditions in different environments (Cabrera-Bosquet et al., 2009) and in different years (Glenn and Bassett, 2011; Glenn, 2014; Merah et al., 2001). In our study, the lack of variation observed after 15 days of treatment and held throughout the recovery period could be due to the short considered period of two weeks that did not allow for any significant change in the percentage of ash. We are aware that also a longer period of study would be required, perhaps annual or seasonal, in order to measure new stem growth and thus, find differences.

3.2. Molecular analysis of the drought stress response

The response to drought stress of two supposed target genes, the dehydrin *ppa005514m* and the gene encoding the LEA protein *ppa008651m*, was analysed throughout the drought and recovery periods. Both genes are related to one of the ABA synthesis pathways (Allagulova et al., 2003; Battaglia et al., 2008; Leida et al., 2012). In addition, two TFs were analysed including the bZIP TF *ppa013046m* belonging to the S group of the bZIP family (Jakoby et al., 2002) and related to proline synthesis (Kiran and Abdin, 2012; Lee et al., 2006), and *ppa012373m* which encodes an A20/AN1 zinc-finger protein involved in responses to different abiotic stresses as cold, salt, dehydration and bud dormancy entrance (Giri et al., 2011; Leida et al., 2012; Mukhopadhyay et al., 2004). The gene expression patterns were studied in young tissue from the phloem and roots by RT-qPCR in 'Garnem', 'Tri-hybrid-3' and OP-'P.2175' plants. A higher response at the root level was observed in comparison to the phloem for the TFs and dehydrin genes, but not the LEA gene, whose expression in OP-'P.2175' at 15 day of treatment was similar both phloem and root tissue (Fig. 4). These observations demonstrate that the primary response to drought stress occurs in the root by a lack of water in the soil (Aguado et al., 2014; Wisniewski et al., 2004). This trend was observed in all four of the studied genes in both tissues and in all genotypes. The gene expression levels were the highest in OP-'P.2175' and the lowest in 'Garnem' (Fig. 4).

3.2.1. Expression profiles of the TFs.

The expression levels of the *ppa012373m* gene, encoding the A20/AN1 zinc-finger protein, changed slightly throughout the stress period in phloem tissue in all genotypes. Comparing the expression levels between each day of treatment to day 0 (control expression level) in phloem, significant differences were found in 'Tri-hybrid-3' (3-fold higher) and in OP-'P.2175' (2-fold higher) on 15 days of treatment and in 'Garnem' genotype (1.6-fold higher) on 15 days after recovery (Fig. 4A). Only significantly differences were observed among genotypes on 15 days of treatment in phloem tissue, being 'Tri-hybrid-3' expression significantly different from 'Garnem' expression (2-fold higher) (Supplementary table S3). In root tissue, both 'Garnem' and 'Tri-hybrid-3' did not show significant differences in *ppa012373m* expression throughout

the experiment compared to the control level (day 0), although an increase of expression was observed on day 15 of the stress period and on day 15 of the recovery period (Fig. 4B). Expression peaks were observed in OP-'P.2175' roots on day 15 of the treatment (12-fold increase) and 15 days after recovery (3-fold increase) compared to day 0 levels, showing significant differences in both cases (Fig. 4B). Among genotypes, significant differences were found along the days of treatment (Supplementary Table S3). So, the gene expression rate in 'OP-P.2175' was significantly different to the rates in 'Garnem' at 10 days of treatment. At 15 days of treatment, gene expression values in OP-'P.2175' were significant different to rates reached in 'Garnem' and 'Tri-hybrid-3'. During the recovery period, 'Tri-hybrid-3' was the genotype with a significant higher gene expression rate compared to the other genotypes at 10 days of recovery. Finally, after 15 days of recovery, the gene expression values in hybrids were significant higher than the gene expression rate in 'Garnem' (Supplementary table S3). The gene encoding the A20/AN1 zinc-finger protein, *ppa012373m*, is homologous to the *SAP-8* gene of *Vitis vinifera, P. mume* and *Malus domestica*. In these species, this gene belongs to *Stress Associated Protein (SAP)-like* (SAP) family, which is characterized by the presence of A20/AN1 zinc-finger domains. SAP-like proteins have also been described in other species such as *Populus trichocarpa* (Jin et al., 2007), *Oryza sativa* (Vij and Tyagi, 2006) and *Aeluropus littoralis* (Ben Saad et al., 2010), suggesting that they are involved in the response to different stresses such as low temperatures, drought and salinity. The overexpression of different genes belonging to this family in rice (Giri et al., 2011; Huang et al., 2008; Kanneganti and Gupta, 2008; Mukhopadhyay et al., 2004) confirmed its regulatory role in these stresses, showing a higher expression during the early phase of the stress response. In our experiment, the higher expression at 10 and 15 days of treatment in this TF would suggest its role in acclimatization phase. In addition, Ben Saad et al., (2010) observed that the upregulation of several *LEA* genes in *AlSAP* transgenic lines suggesting that *SAP* gene would active the expression of these target genes. Mukhopadhyay et al. (2004) suggested a role of the *OSISAP1* gene in preventing damages caused by stress and also promote a better recovery after the stress period. This

hypothesis could also be valid for this experiment and would explain the trend followed by 'Tri-hybrid-3' and OP-'P.2175' in both tissues (Fig. 4).

The *bZIP* gene, *ppa013046m*, is orthologue to the *bZIP3 cis-element-binding factor 1* gene from *M. domestica* and *AtbZIP53* from *A. thaliana*. These TFs belong to the S group described by Jakoby et al. (2002), and they function as transcriptional activators of the *ProDH* gene. Signals deriving from H_2O_2 and the ABA-dependent synthesis pathway during drought and salinity stress activate the *P5CS* gene, which induces the accumulation of proline (Saradhi et al., 1995; Strizhov et al., 1997; Yoshiba et al., 1997). During the first hours of rehydration, the metabolism of proline (which accumulated during stress) to glutamate is regulated by the *ProDH* gene (Satoh et al., 2004; Yoshiba et al., 1997). In our study, the *ppa013046m* gene did not show significant differences in 'Garnem' both phloem and root tissues (Fig. 4C and D), as well as 'Tri-hybrid-3' (Fig. 4C and D). Nevertheless, the *bZIP* gene was significant under-expressed in 'Tri-hybrid-3'at 15 day of recovery compared to control expression level in root tissue (Fig. 4D). During the stress period, *ppa013046m* expression was significantly higher in the roots from OP-'P.2175' (Fig. 4D), reaching levels 3-fold higher at 10 days and 4-fold higher at 15 days compared to day 0, but not in phloem tissue (Fig. 4C). However, the level expression of the TF was significantly lower in phloem from OP-'P.2175' after 15 days of the recovery period (Fig. 4C). Among genotypes for each day of treatment, no significant differences were found in phloem (Supplementary table S3). While, in the roots, the level expression of *ppa013046m* was significant higher in OP-'P.2175' than in 'Garnem' at 10 days of treatment and significant higher than 'Garnem' and 'Tri-hybrid-3' at 15 days of drought stress (Supplementary table S3). Since *ProDH* gene is active during the first hour of rehydration, we would expect that its transcriptional activator would also be expressed under these conditions. On the contrary, our results were not consistent with the assumptions discussed above. A possible reason could be due to other metabolic factors involved in the induction of the *ppa013046m* gene during the stress period that require consideration in the future. Even if it seems not to be involved in rehydration process, the higher expression in OP-'P.2175' makes it

409 useful as a marker of drought stress; even if the reasons and the mechanism that stand below are
410 still to be unravelled.
411 In spite of the most of reports studying TFs expression had been done at short-term stages of the
412 drought response (Giri et al., 2011; Huang et al., 2008; Kanneganti and Gupta, 2008;
413 Mukhopadhyay et al., 2004), Su et al., (2013) observed the overexpression of different TFs at
414 long-term experiment, demonstrating the important role of TFs, not only as transcriptional
415 activators of target genes at early response to drought, but during the acclimatization phase.

416 *3.2.2. Expression profiles of the target genes.*
417 The expression levels increased both in the dehydrin gene (*ppa005514m*) and in the gene
418 encoding the LEA protein (*ppa008651m*) throughout the stress period, reaching an expression
419 peak by 15 days of treatment, and their levels dropped significantly during the recovery period
420 (Fig. 4E, F, G, and H). The same trend was observed in all genotypes, both in phloem and root
421 tissues. These two genes belong to the LEA protein family (Allagulova et al., 2003; Battaglia et
422 al., 2008), which plays a main role in acclimatization and the adaptive response to stress
423 processes by conferring tolerance under drought conditions, low temperatures and osmotic
424 stress (Battaglia et al., 2008; Xiao et al., 2007). The expression of *LEA* genes is not specific for
425 a particular tissue. These genes can be expressed in both leaves and roots or stems and even in
426 the cotyledons (Hong-Bo et al., 2005).
427 The dehydrin expression levels (*ppa005514m*) showed statistically significant increases in
428 phloem tissue at all stages of the experiment in comparison to day 0 (control), while in root
429 tissue the expression levels increased significantly only during the stress period decreased
430 dramatically during recovery (Fig. 4E and F). In 'Garnem', the expression level of *ppa005514m*
431 was significantly 2.4-fold higher at 10 and 15 days of treatment in comparison to day 0 in
432 phloem (Fig. 4E). In root tissue, 'Garnem' increased significantly the expression of the dehydrin
433 genbeing 24-fold higher on day 10 and 25-fold higher at 15 days of treatment in comparison to
434 control (Fig. 4F). The *ppa005514m* expression in 'Trihibrid-3' was significantly higher (6-fold)
435 at 15 days of treatment in phloem (Fig. 4E). In the root tissue, the expression level was

436 significanlty 17-fold higher at 15 days (Fig. 4F). Meanwhile, OP-'P.2175' showed a 2-fold
437 higher expression in phloem by 10 days and 5-fold higher by 15 days of drought period (Fig.
438 4E). After 15 days, *ppa005514m* expression was 23-fold higher in roots (Fig. 4F). During the
439 recovery period, there were only significant differences in *ppa005514m* expression levels in
440 phloem. The dehydrin expression was less than that on day 0 in OP-'P.2175' by 10 days and in
441 'Garnem' at two weeks (Fig. 4E). Among genotypes, significant differences were found at 15
442 days of treatment, when the dehydrin expression in 'Tri-hybrid-3' was significantly different to
443 the expression in 'Garnem' in the phloem (Supplementary table S3), as well as in root tissue at
444 15 days, when 'Tri-hybrid-3' and 'OP-'P.2175' genotypes presented a significant higher
445 expression levels than 'Garnem' (Suplementary table S3). In the same tissue, *ppa005514m*
446 expression was significantly higher in 'OP-'P.2175' than the others genotypes at 15 days of
447 recovery (Supplementary table S3). The *ppa005514m* gene encodes a dehydrin belonging to
448 group 2, also known as D-11 group (Battaglia et al., 2008). Dehydrins have been studied in
449 woody plants (Artlip and Wisniewski, 1997; Bassett et al., 2009; Velasco-Conde et al., 2012;
450 Vornam et al., 2011; Wisniewski et al., 2009, 2006), confirming the existence of a direct
451 relationship between the accumulation of dehydrins in tissues and tolerance to abiotic stresses.
452 Artlip et al. (1997) identified the *ppdhn1* gene and they demonstrated its protective role during
453 dehydration caused by low temperatures and drought stress in *P. persica* and showed its
454 induction by ABA. Wisniewski et al. (2006) observed that the accumulation of *ppdhn1* in peach
455 bark was higher than in leaves under drought stress. Moreover, as in our work, Wisniewski et al.
456 (2006) found that after a week of severe drought stress, the accumulation of *ppdhn1* transcripts
457 decreased in bark when the plants recovered their water status (Wisniewski et al., 2006). On the
458 contrary, under low-temperature conditions, *ppdhn1* transcripts did not accumulate in root
459 tissues due to the minimum temperature changes that the roots might suffer throughout the
460 seasons as compared to the damages suffered in buds where *ppdhn1* accumulation was higher
461 (Wisniewski et al., 2004). So this gene is supposed to be involved in drought and low
462 temperature tolerance mechanisms. These observations are consistent with the results describing
463 the dehydrin tendency in the tissues studied in our work. Roots would be more sensitive to the

lack of water in the substrate, resulting in higher gene expression levels in root tissue than in phloem. This condition is also true for the TFs analysed above. It was observed that the expression of 24-kd dehydrin was stronger in drought-tolerant plants than in sensitive plants at a higher water potential (Lopez et al., 2001, 2003), as it is consistent with our findings. 'Tri-hybrid-3' and OP-'P.2175'registered higher LWP and dehydrin expression levels than 'Garnem' (Fig. 1A and 6), suggesting that the accumulation of dehydrin would be related to the better drought tolerance showed by the 'Garnem' progeny.

The gene encoding the LEA protein (*ppa008651m*) was identified in a transcriptomic study of genes subjected to low temperatures in peaches (Ogundiwin et al., 2008). This gene is homologous to the gene encoding a D-29 LEA protein belonging to the 3B group described by (Battaglia et al., 2008). When the relative expression of the *ppa008651m* gene was analysed, significant differences were found in comparison to day 0 levels both in phloem and root tissues throughout the stress period, and on 10 days after recovery (Fig. 4G and H). For the 'Garnem' genotype, the expression showed a peak at 15 days of stress in phloem with a value 53-fold higher than control levels (Fig. 4G), whereas the expression values were 31- and 26-fold higher in root tissue on 10 and 15 days of the stress period, respectively (Fig. 4H). For the two hybrids, the highest expression level was reached on day 15 of the stress period, highlighting OP-'P.2175' on the other genotypes with a value 311-fold higher in phloem (Fig. 4G) and 130-fold higher in roots with respect to the reference status at day 0 (Fig. 4H). During the recovery period, *ppa008651m* gene expression dropped to similar levels as those on day 0, showing statistical differences at 10 days for phloem in 'Garnem' (Fig. 4G) and in 'Tri-hybrid-3' genotype in both phloem (Fig. 4G) and root tissues (Fig. 4H). Significant differences were found when the *LEA* gene expression levels were compared among genotypes. So, this gene expression was significantly higher at 10 and 15 days of treatment in 'OP-'P.2175' than in 'Garnem' and 'Tri-hybrid-3', as well as significantly higher at 10 days of recovery in 'Garnem' than in the other genotypes in the phloem (Supplementary table S3). Furthermore, its expression level was significantly higher at 15 days of drought stress in OP-'P.2175' than in 'Garnem' and 'Tri-hybrid-3' in root tissue. It is noteworthy that the control level expression in 'Tri-hybrid-3'

was significantly higher than in the others genotypes in this same tissue (Supplementary table S3). Various studies showed the relationship of group 3 LEA proteins in the response to abiotic stress. For example, the *Hva1* gene, identified in barley, confers drought tolerance in transgenic rice, due to its protective role of the cellular membrane (Babu et al., 2004). In rice, the *OsLEA3-1* gene was also identified and overexpressed showing that the transgenic plants improved their drought tolerance and maintaining the yield (Xiao et al., 2007). In addition, Leida et al. (2010) found that the *ppa008651m* gene was associated with dormancy in peaches under low-temperature conditions. In our experience, we verified that *ppa008651m* expression is activated not only under low temperatures, but that it is also induced by dehydration caused by drought.

4. CONCLUSIONS

From the physiological and molecular ~~results obtained and considering that~~data under our specific experimental conditions, the two hybrid genotypes showed a better adaptive response to drought than the 'Garnem' genotype, this is especially true for OP-'P.2175'. All genes studied had the maximum expression level in root tissue (Fig. 4), while LWP and gs reached the minimum value at 15d of treatment (Fig. 1), confirming a drought stress response. ~~In our work, we tested t~~The genes encoding the LEA and dehydrin proteins ~~that~~ can be proposed as biomarkers in the selection of more tolerant plants within a drought tolerance breeding program. In this work, we demonstrated their correlation by showing higher expression in the best adaptive response plants. It would be interesting to confirm our results also in other species and hybrids. On the other side, the gene expression of the TFs tested was confirmed at long-term stage. Nevertheless, additional experiments are required in order to test their involvement during the early hours of exposure to drought stress.

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805

806 **TABLE**

807 **Table 1.** Primer sequences used in the RT-qPCR analysis.

Primer Name	Gene	5' to 3' Sequence	Primer Reference
Dehydrin F	*ppa005514m*	GTACTCTCATGACACCCACAAAACTAC	Leida et al. 2012
Dehydrin R		CCCGGCCCCACCGTAAGCTCCAGTT	
LEA protein F	*ppa008651m*	GCAAAAGGTAGGGCAAACAG	Leida et al. 2012
LEA protein R		TGGCTTTGCTTCTTTGGTCT	
Zn-Finger F	*ppa012373m*	ACACAGGCTTCCTCTACTCCATCTTT	Leida et al. 2012
Zn-Finger R		GAACCCTCATTCCGAGACATTTATCAG	
ppn070g03 F	*ppa013046m*	GGGTTGAAACACCCAAAAGA	
ppn070g03 R		GCGATTCGACAACATCCTCT	
Actin F	*ppa007242m*	CAGATCATGTTTGAGACCTTCAATGT	
Actin R		CATCACCAGAGTCCAGCACAAT	

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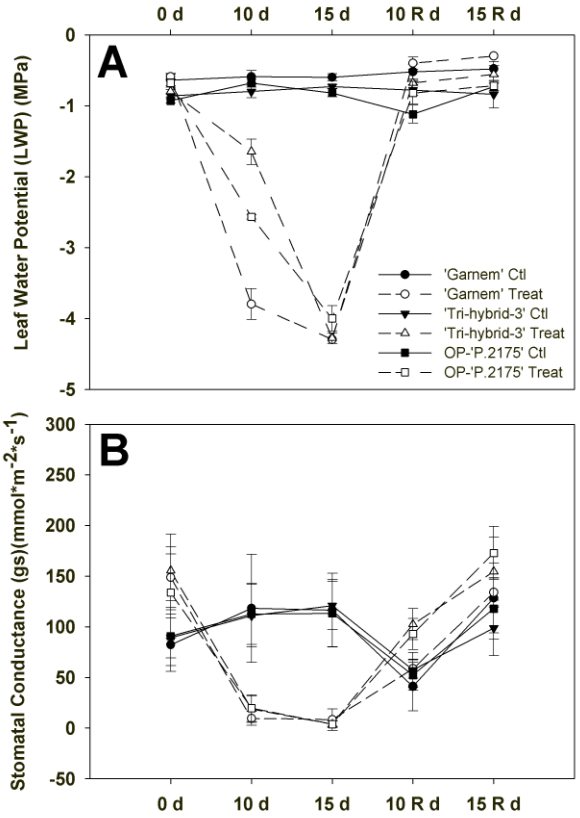
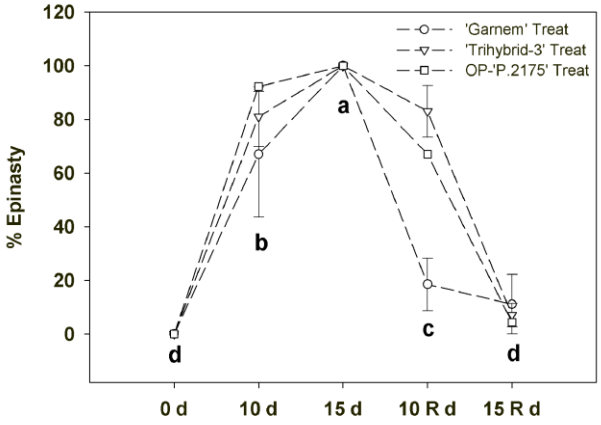
809

810 **FIGURES**



811

812 **Fig. 1.** Leaf Water Potential (LWP) (A) and stomatal conductance (gs) (B) during the drought
813 experiment for the studied genotypes. Continuous lines indicate water supplied plants while dot
814 lines indicate hydric conditions in plants under drought treatment. (d = days, R= Recovery).
815 Error bars represent the standard error of the mean.



816

Fig. 2. Leaf epinasty percentage during the experiment for the genotypes under drought conditions. Similar letter values indicate no significant difference ($p \leq 0.05$) following Tukey's post-hoc test. (d = days, R = Recovery). Error bars represent the standard error of the mean.

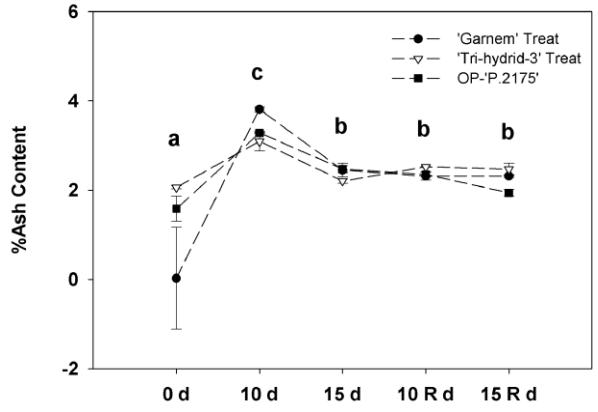


Fig. 3. Ash content percentage in wood tissue during the experiment for the genotypes under drought conditions. Similar letter values indicate no significant difference ($p \leq 0.05$) following Tukey's post-hoc test. (d = days, R = Recovery). Error bars represent the standard error of the mean.

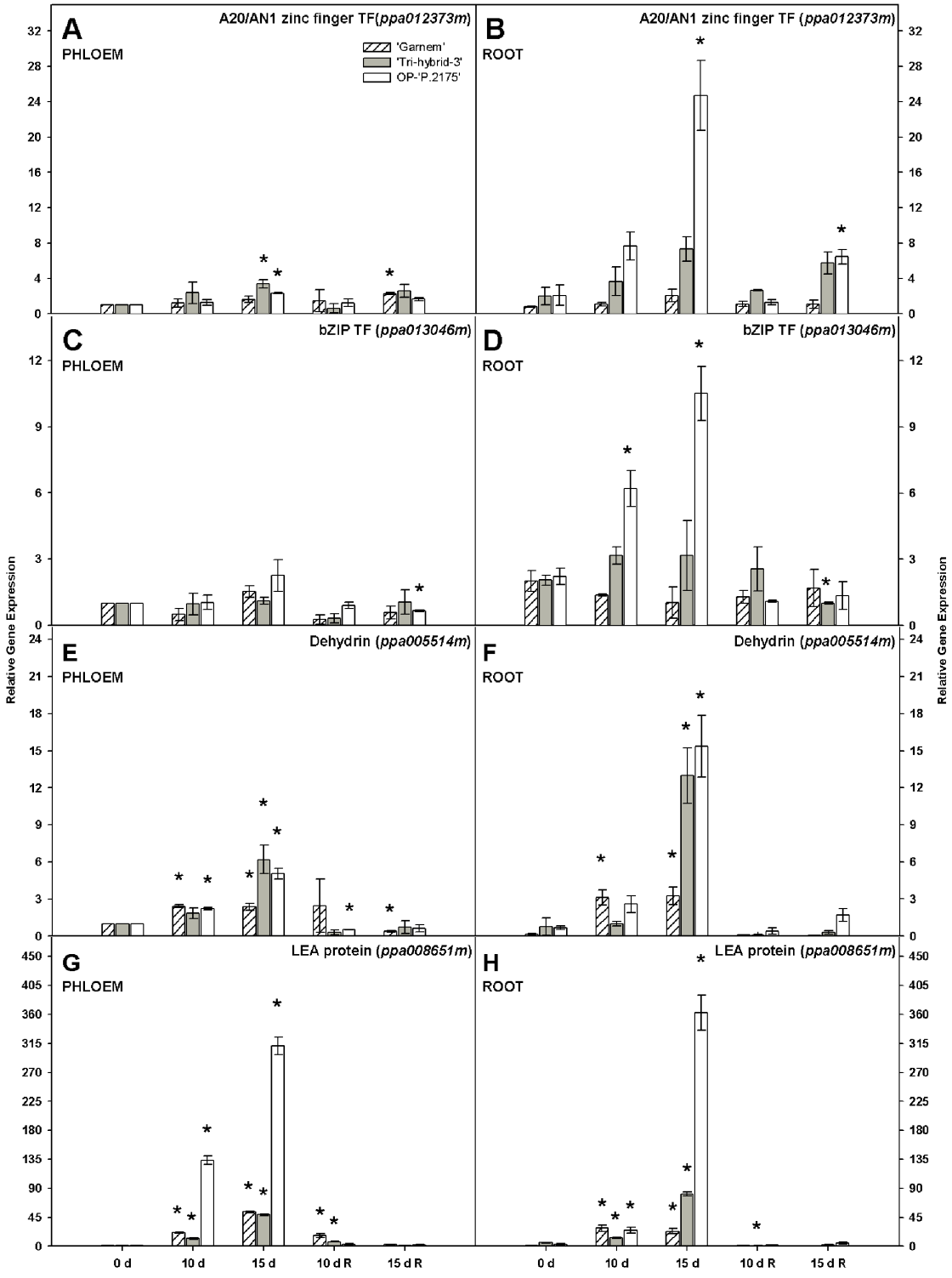


Fig. 4. Relative expression of the A20/AN1 zinc finger TF (*ppa012373m*)(A and B); the bZIP TF (*ppa013046m*) (C and D); the dehydrin (*ppa005514m*) (E and F); and the LEA protein (*ppa008651m*) (G and H). Expression levels were compared to the *actin* gene. The relative value of 1 was assigned to the phloem sample on day 0 (control day value). Data show the

average relative expression of two biological samples with three technical replicates each one. Asterisks indicate significantly different expression values ($p \leq 0.05$) for each genotype with respect to day 0 following the Student's t-test. (d = days, R = Recovery). Error bars represent the standard error of the mean.

SUPPLEMENTARY DATA LEGEND

Supplementary Data Sheet S1. RNA isolation protocol by Meisel et al. (2005) with some modifications (Chang et al., 1993; Salzman et al., 1999; Zeng and Yang, 2002).

Supplementary Table S1. Daily environmental data along the experimental period.

Supplementary Table S2. ANOVA results from Leaf Water Potential (LWP) and Stomatal Conductance (gs) during the drought experiment for the studied genotypes. Same letter values indicate a no significant difference ($p \leq 0.05$) following Tuckey's post hoc test. (d=days, R= Recovery).

Supplementary Table S3. ANOVA results from Relative Gene Expression during the drought experiment for the studied genotypes. Same letter values indicate a no significant difference ($p \leq 0.05$) following Tuckey's post hoc test among genotypes for each tissue and each day of treatment. (d=days, R= Recovery).

Table 1. Primer sequences used in the RT-qPCR analysis.

Primer Name	Gene	5' to 3' Sequence
Dehydrin F	*ppa005514m*	GTACTCTCATGACACCCACAAAACTAC
Dehydrin R		CCCGGCCCCACCGTAAGCTCCAGTT
LEA protein F	*ppa008651m*	GCAAAAGGTAGGGCAAACAG
LEA protein R		TGGCTTTGCTTCTTTGGTCT
Zn-Finger F	*ppa012373m*	ACACAGGCTTCCTCTACTCCATCTTT
Zn-Finger R		GAACCCTCATTCCGAGACATTTATCAG
ppn070g03 F	*ppa013046m*	GGGTTGAAACACCCAAAAGA
ppn070g03 R		GCGATTCGACAACATCCTCT
Actin F	*ppa007242m*	CAGATCATGTTTGAGACCTTCAATGT
Actin R		CATCACCAGAGTCCAGCACAAT

Figure1

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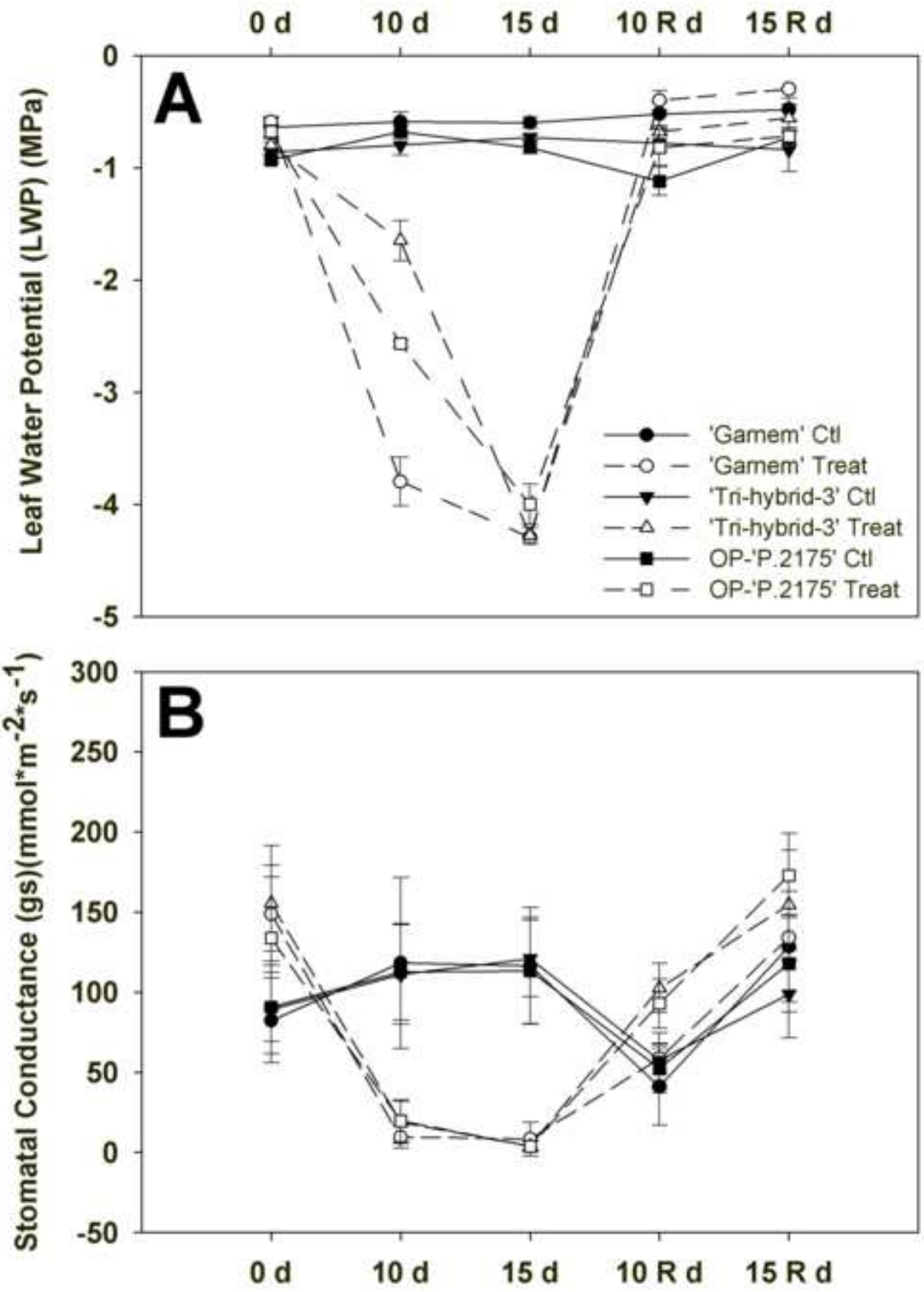

Figure 2

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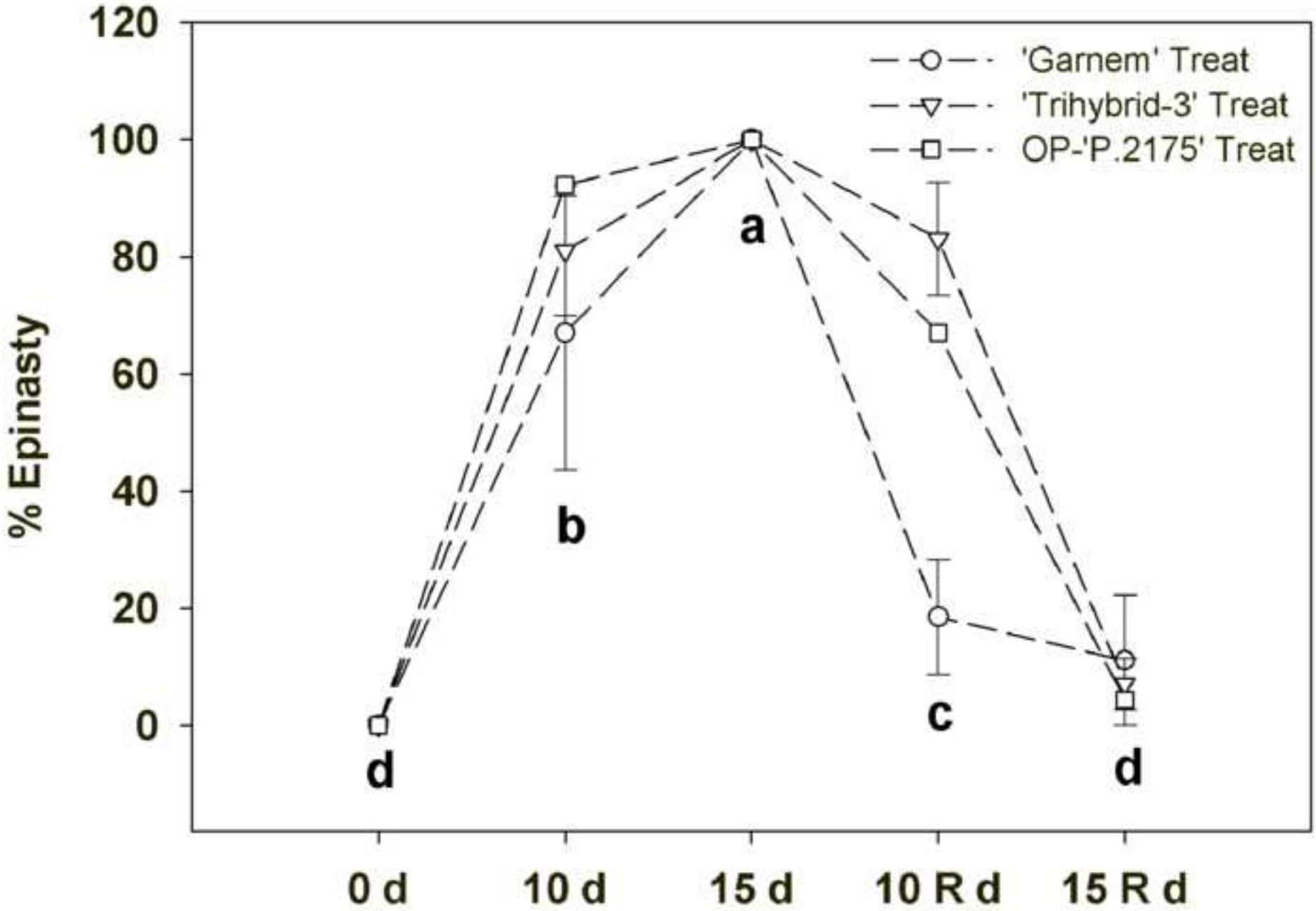

Figure 3

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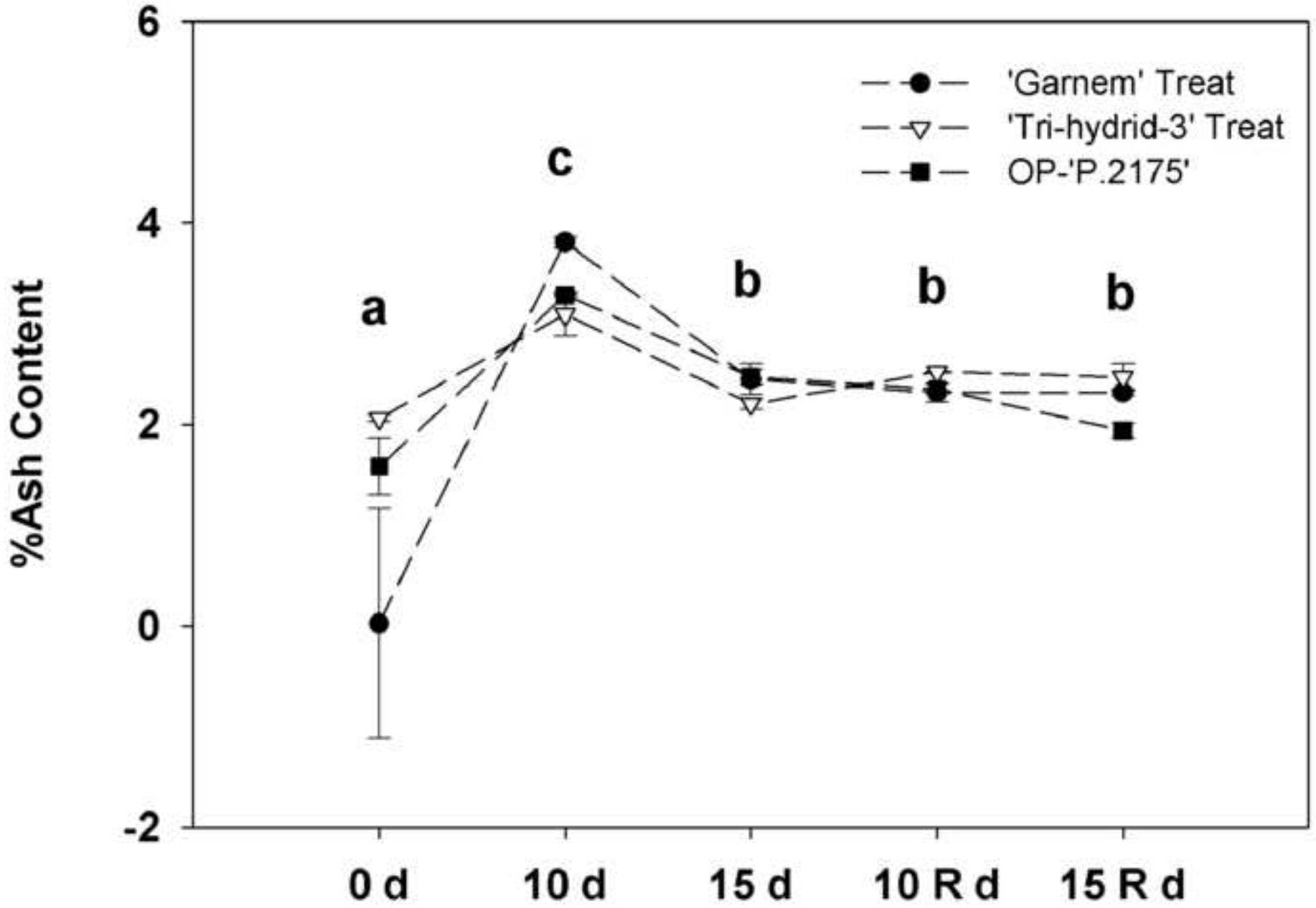

Figure 4

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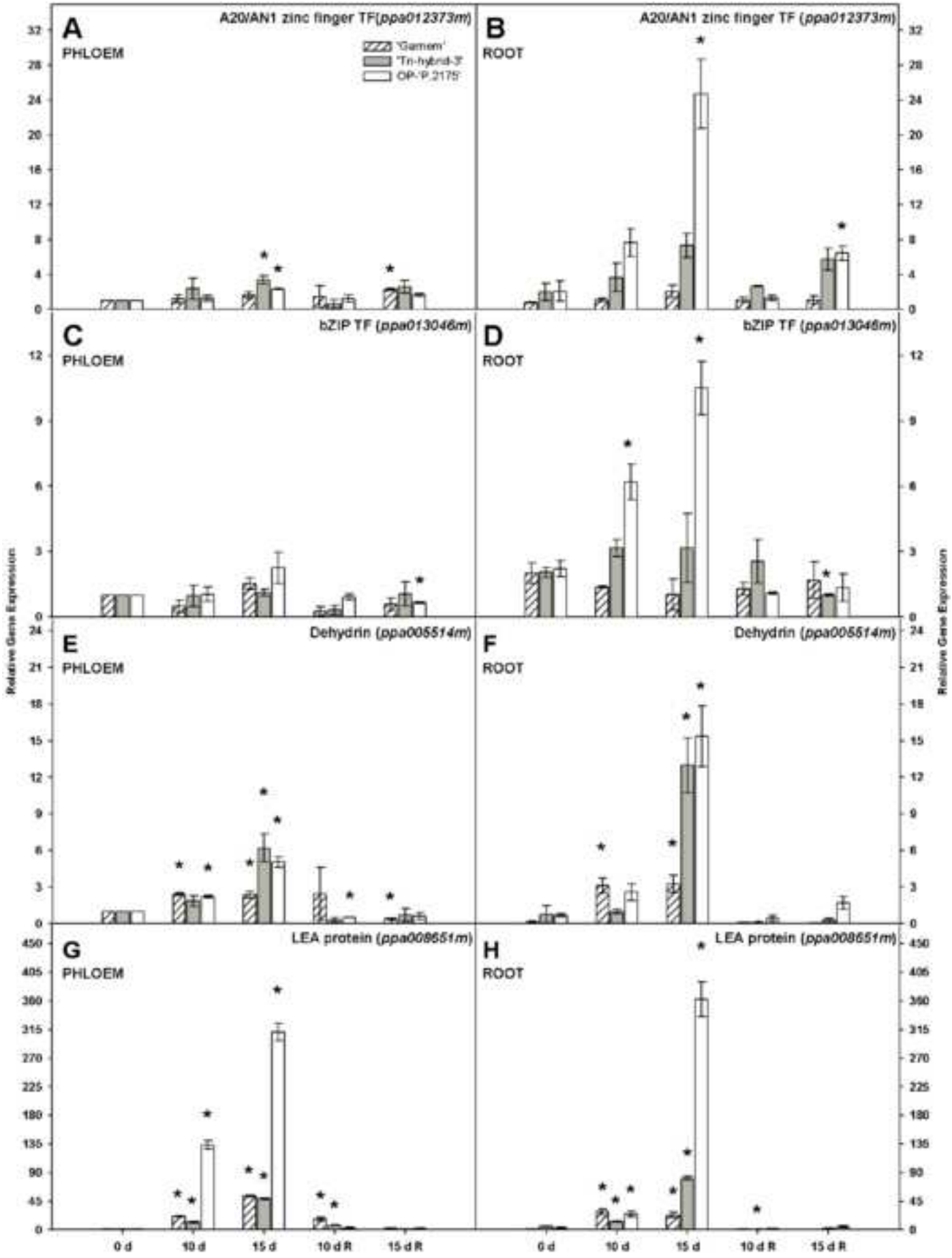

Supplementary TABLE S1

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Supplementary TABLE S3

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Supplementary DATA SHEET S1

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